Aus dem Institut für Veterinärbiochemie und Molekularbiologie
der Vetsuisse-Fakultät Universität Zürich

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The Tumour Suppressor Protein Flap Endonuclease 1 Interacts Physically with the Heterotrimeric Checkpoint Protein Complex Rad9, Rad1 and Hus1

INAUGURAL-DISSERTATION

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Für meine Eltern
Ursula und Christian

Und meine Geschwister
Simon und Sarah

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1 Summary

1.1 English

To guarantee a successful function and reproduction a cell is faced with the challenging task of maintaining the stability of its genome and replicating its DNA. Throughout evolution many mechanisms have been established to guarantee genomic stability from generation to generation. The cell possesses very sophisticated cell cycle checkpoint pathways to survey the different phases of the cell cycle including cell division and to detect DNA damages. The heterotrimeric protein complex Rad9, Rad1 and Hus1, called the '9-1-1 complex', is an important key player in such checkpoint pathways. The '9-1-1 complex' exhibits structural similarity with the homotrimeric clamp formed by proliferating cell nuclear antigen (PCNA). PCNA is known to form a complex with the tumour suppressor protein flap endonuclease 1 (Fen1). The goal of this study was to characterize the interaction of the '9-1-1 complex' with Fen1, an enzyme involved in DNA replication and various repair mechanisms. For this purpose the human '9-1-1 complex' was isolated by co-expressing the three baculovirus encoding the recombinant Rad9, Rad1 and Hus1 in Sf9 cells. In pull down experiments and by Far Western Blot analysis it was shown that Fen1 physically interacts with the '9-1-1 complex' from Sf9 cell extract as well as with the purified '9-1-1 complex'. Furthermore Fen1 binds to each of the three monomers Rad9, Rad1 and Hus1. The binding seems to occur through the Fen1 C-terminus. These findings are also interesting form a evolutionary point of view since the Archaea homologue of Fen1 is missing its C-terminus and no checkpoint proteins are found in this early organisms. Previous studies from our laboratory documented that acetylation of Fen1 is increased upon UV damage. To test if this has an effect on the binding to the '9-1-1 complex' a pull down experiment with *in vitro* acetylated Fen1 was performed. In summary, the results presented in this work support the hypothesis that the '9-1-1 complex' has an important role in DNA repair.

1.2 German

Um eine erfolgreiche Funktion und Fortpflanzung der Zelle zu garantieren, ist diese mit der herausfordernden Aufgabe konfrontiert, die Stabilität ihres Genoms zu wahren und ihre DNA zu kopieren. Im Laufe der Evolution sind viele Mechanismen entstanden, die mithelfen die Stabilität der Erbinformation zu garantieren. Die Zelle verfügt über sehr ausgeklügelte Zellzykluskontrollsysteme, um die verschiedenen Phasen des Zellzyklus inklusive Zellteilung zu überwachen und um DNA Schäden festzustellen. Der heterotrimere Proteinkomplex Rad9, Rad1 and Hus1, bezeichnet als den '9-1-1 Komplex', hat eine Schlüsselstelle in solchen Kontrollsystemen. Der '9-1-1 Komplex' hat strukturelle Ähnlichkeiten mit dem ringförmigen Homotrimer Proliferating Cell Nuclear Antigen (PCNA). Von PCNA weiss man, dass es mit dem Tumorsuppressorprotein Flap endonuclease 1 (Fen1) einen Komplex bildet, dessen Rolle in der DNA Replikation und verschieden Reparaturmechanismen bekannt ist. Das Ziel dieser Dissertation war es, die Interaktion zwischen dem '9-1-1 Komplex' und Fen1 zu charakterisieren. Der '9-1-1 Komplex' wurde aus Sf9 Zellen isoliert, die mit 3 Baculoviren infiziert worden waren. Die Viren enthielten die DNA von den rekombinanten Proteinen Rad9, Rad1 und Hus1 und wurden in den Zellen überexprimiert. Mittels 'pull down' Experimenten und 'Far Western Blot' Analysen konnte gezeigt werden, dass Fen1 physisch sowohl mit dem '9-1-1 Komplex' aus Extrakt von Sf9 Zellen als auch mit gereinigtem '9-1-1 Komplex' interagiert. Fen1 bindet einzeln an jede der drei Untereinheiten Rad9, Rad1 und Hus1. Die Bindung geschieht über den C-terminus von Fen1. Dieses Ergebnis ist auch vom evolutionären Standpunkt aus interessant, hat nämlich weder das Fen1 Homolog in Archaea einen C-terminus noch wurden in diesen Organismen Checkpoint Proteine gefunden. Frühere Studien aus unserem Labor zeigten, dass die Azetylierung von Fen1 nach einer UV Schädigung ansteigt. Um zu testen, ob die Azetylierung von Fen1 einen Einfluss auf die Interaktion mit dem '9-1-1 Komplex' hat, wurde ein 'pull down' Experiment mit *in vitro* azetyliertem Fen1 durchgeführt. Die Ergebnisse dieser Arbeit bestärken die Hypothese, dass der '9-1-1 Komplex' eine wichtige Funktion in der DNA Reparatur hat.

2 Introduction

2.1 Genomic stability and the cell cycle

All necessary information for the key characteristics of life is stored on the DNA in each cell nucleus. Because DNA is a chemically highly reacting molecule it is a dedicated target for different kinds of chemical and physical substances. Not only environmental influences but also misincorporation of nucleotides during DNA replication can cause DNA damages or lead to incorrect DNA sequence or structure. To guarantee a successful function and reproduction a cell is faced with the challenging task of maintaining the stability of its genome and replicating its DNA. Over billion of years the evolution developed and conserved different strategies to overcome these problems, enormous machineries of proteins coordinate these fundamental and complicated events. Despite the evolutionary distance the functions of proteins involved and the mechanisms at the basis of these events are strikingly similar in prokaryotes, archeas and eukaryotes, the three kingdoms of life (Tye, 2000). In multicellular organism also the communication and the coordination among the cells is very important. The loss of ability of cells to communicate and act selflessly as a part of an integrated whole can lead to severe disorders, such as cancer and hereditary diseases.

The cell cycle is divided in four phases: G1-phase (gap 1), the phase of actual cell activity. This phase is followed by the S-phase (synthesis) where the genomic DNA is copied. The third phase is called G2-phase (gap 2), a short interval in which cell division is prepared and initiated. The M-phase (mitosis) unites all the different stages of mitosis: Condensation of the chromosomes, alignment of the homologous chromosomes in the central plane of the cell, separation of the chromosomes into two equivalent sets of genomic DNA copies and finally cell division. Differentiated cells that cannot divide any longer proceed into an additional phase called G0 (gap 0) corresponding to a constant G1-phase.

Throughout evolution many mechanisms have been established to guarantee genomic stability from generation to generation. Firstly, DNA replication is performed by DNA polymerases and auxiliary proteins with high fidelity, among them flap endonuclease 1 (Fen1) (Harrington and Lieber, 1994a) one of the key players of this thesis. Secondly, the cell possesses very sophisticated cell cycle checkpoint pathways to survey the different phases of the cell cycle including cell division and to detect DNA damages. The checkpoint protein complex Rad9, Rad1 and Hus1, the '9-1-1 complex' (al-Khodairy et al., 1994), the other protagonist of this work, is known to be involved in the checkpoint cascade. The cellular answer provoked by DNA damage includes the slowing down of the cell cycle progression and stalling it at the G2/M transition (reviewed in Foiani et al., 2000), thus providing time to repair damage through different repair mechanism (reviewed in Christmann et al., 2003) such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair and double-strand break repair (DSBR) or complete replication before the cell enters into mitosis.

2.2 Flap endonuclease 1 (Fen1)

The structure-specific flap endonuclease 1 (Fen1) (Harrington and Lieber, 1994a) is a key enzyme for maintaining genomic stability. It plays an important role in DNA replication and repair (reviewed in Henneke et al., 2003a). During DNA replication, it participates in the removal of the displaced RNA-DNA primers during Okazaki fragment maturation (Hubscher and Seo, 2001). In DNA repair, it has been implicated in long patch base excision repair and seems to be required for non-homologous end joining of double-strand DNA-breaks (DSB). Fen1 is also claimed to be a tumour suppressor when it was shown that Fen1 haploinsufficiency in mice leads to rapid tumour progression (Kucherlapati et al., 2002, Fodde and Smits, 2002).

2.2.1 *Structure-function and post-translational modifications of Fen1*

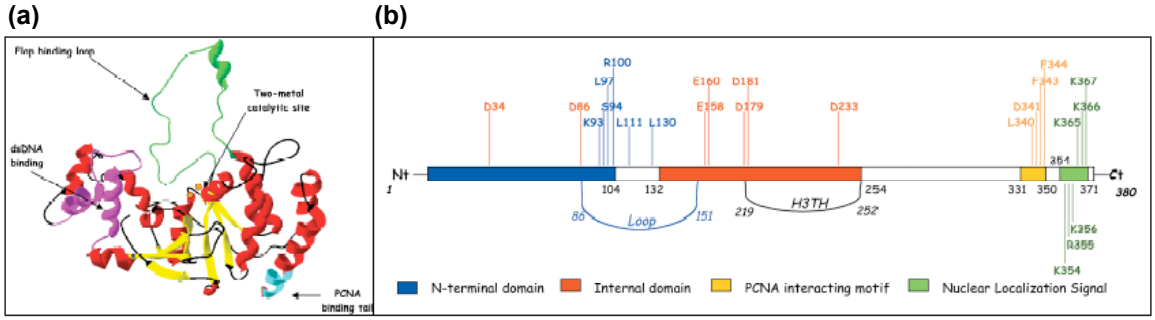

Figure 1. Structure and function of flap endonuclease 1 (Fen1). **(a)** Model building of a 3D structure of human Fen1 according to Swiss-Pdb Viewer. **(b)** Functional domains and important amino acids of human Fen1 (Reproduced from Henneke et al., 2003a).

The crystal structure of Fen1 and its homologues from different species are available (Hwang et al., 1998, Hosfield et al., 1998, Chapados et al., 2004). Several attempts to solve the structure of human Fen1 (hFen1) were launched not only in the Hübscher laboratory but until now the structure of human Fen1 (*figure 1*) as well as the structure of *Saccharomyces cerevisiae* Rad27p are not yet available.

Based on protein sequence comparison and biochemical assays, two major conserved motifs, the N (N-terminal) and I (intermediate) motifs, were found to be essential for the nuclease activities of Fen1 (Shen et al., 1998, Harrington and Lieber, 1994b). A third motif at the C-terminal end is involved in the interaction with proliferating cell nuclear antigen (PCNA) (Shen et al., 1998, Stucki et al., 2001, Warbrick et al., 1997). All crystal structures of Fen1 homologues solved to date (Hosfield et al., 1998, Hwang et al., 1998, Ceska et al., 1996, Chapados et al., 2004) show a conserved helical arch located above the globular domain that contains the active site. This flexible loop, in addition to the catalytic site, was shown to be essential for flap cleavage (Storici et al., 2002) and it has been proposed that the 5′ end of the DNA flap could thread through the hole of the loop tracking the length of the 5’ tail (Murante et al., 1995). Recent data suggested that the substrate specificity of Fen1 is conserved in evolution in

an extraordinary way and that this conservation is due to the structural similarity of the hydrophobic wedge in general and the extrahelical 3'flap pocket in particular (Friedrich-Heineken and Hubscher, 2004). Interaction with an extrahelical 3'nucleotide helps to position the flap precisely so that an efficient cleavage of DNA double-flap structure at a precise site can occur and thereby a ligatable nick for DNA ligase I can be created (Maga et al., 2001).

Fen1 is also a target for two posttranslational modifications *in vivo*: acetylation (Hasan et al., 2001) and phosphorylation (Henneke et al., 2003b). While phosphorylation reduces its endo- and exonucleolytic activities and abrogates its binding to PCNA thus preventing stimulation of Fen1 by PCNA, acetylation through the transcriptional coactivator p300 reduces its DNA binding activity as well as its endo- and exonucleolytic activities.

2.2.2 Fen1 in DNA replication

To maintain the functionality of an organism a continuous reproduction of the cells is necessary. To provide each of the offspring cells with a complete set of information for protein syntheses a new reproduction cycle always starts with the duplication of entire DNA, and this process is called DNA replication.

DNA polymerases (pols) are the key enzymes in this process. All pols share a common activity, they catalyse the synthesis of DNA macromolecules from deoxyribonucleoside triphosphates using chemical energy derived from the hydrolysis of the latter (reviewed in Hubscher et al., 2002).

Once DNA synthesis is initiated at the origin of replication it has to be continued along the DNA double helix. However the double helix is orientated in an antiparallel manner. Determined by the structure of various pols, which can catalyse DNA synthesis exclusively in the 5' → 3' direction, only one strand can be synthesised continuously and is called the leading strand. The opposite mother strand (called the lagging strand) needs to be synthesised in numerous short pieces, called the Okazaki fragments. The two DNA strands form a Y-shaped figure at the point of replication and this arrangement is called the

replication fork. To solve the sterical problem of antiparallel DNA synthesis on double-strand DNA a model was proposed where the lagging strand formed a loop and allowed the pols on both leading and lagging strand to overall progress in the same direction (Sinha et al., 1980). The completion of DNA synthesis at the lagging strand requires the removal of RNA primers from Okazaki fragments before gap-filling synthesis and ligation (Hubscher and Seo, 2001). In this process Fen1 seems to play an important role. Two models of Okazaki fragment processing were proposed (*figure 2*) (reviewed in Henneke et al., 2003a). The polymerase δ displaces the RNA-containing downstream Okazaki fragment, creating a 5'flap. This flap can be processed by Fen1 either in a Dna2-dependent or independent manner depending on the length of the flap (Hubscher and Seo, 2001, Jin et al., 2003). Because the displaced flap by pol δ holoenzyme [pol δ, PCNA, RF-C] is complementary to the template; it is capable of branch migration to form numerous interconverting structures. Of these, Fen1 recognises a double-flap intermediate containing a 1nt 3'-tail with high efficiency and cleaves it to generate a nicked substrate. Cleavage of a conventional flap substrate leaves nicked and gapped structures, which would need gap-filling synthesis by a replicative DNA polymerase before ligation could occur (Maga et al., 2001). Fen1 could be recruited to the site of DNA synthesis by the moving clamp PCNA, which can stimulate Fen1 by stabilising its interaction to the cleavage site (Tom et al., 2000) and must be located below the 5'-flap (Jónsson et al., 1998). The essential PCNA-binding domain was mapped to a region near the basic C-terminus of Fen1 (Stucki et al., 2001).

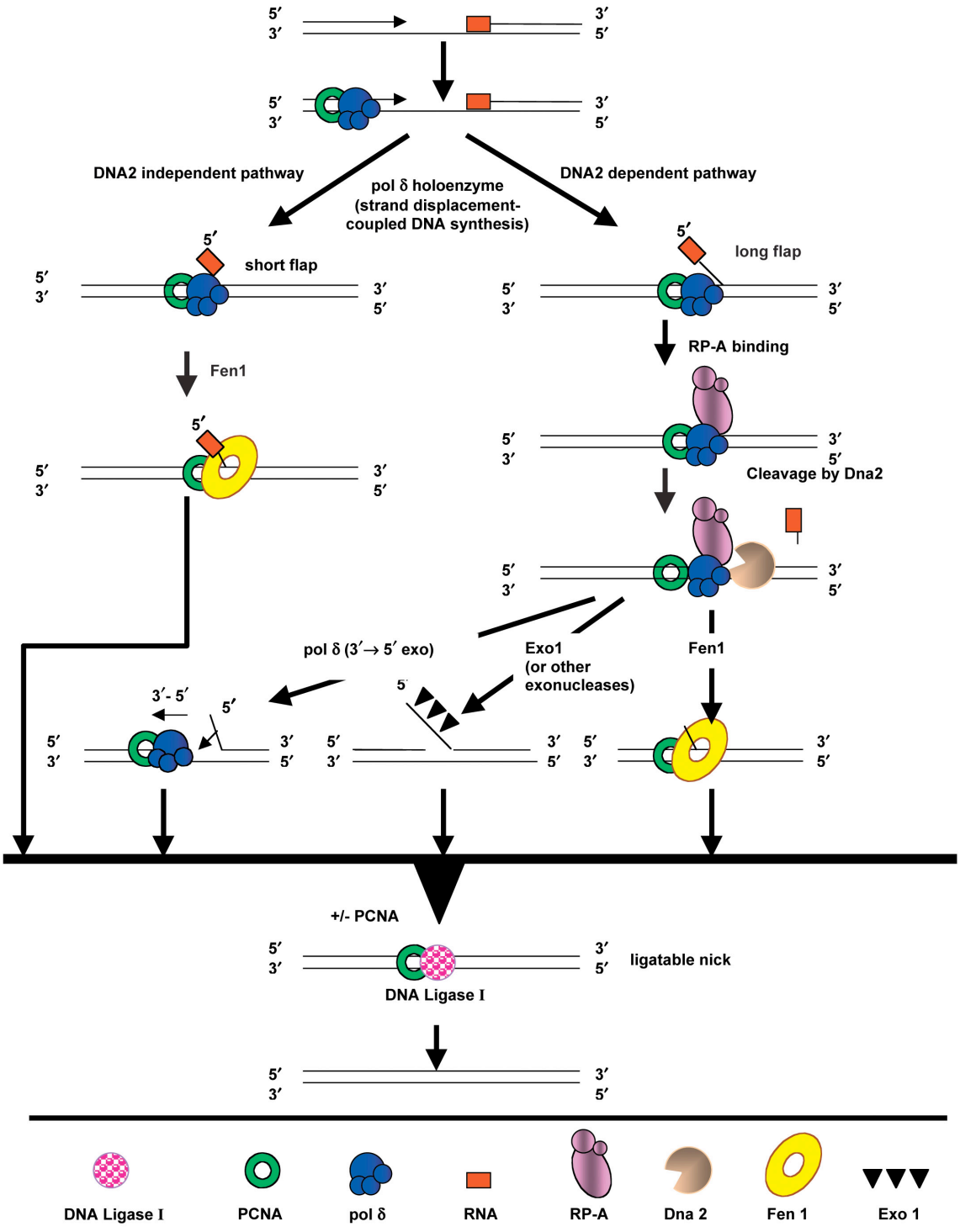

Figure 2. **The various functions of Fen1 in Okazaki fragment processing.** DNA synthesis catalysed by pol δ displaces the RNA-containing downstream Okazaki fragment, creating a 5'-flap. This flap can be processed by Fen1 in either a Dna2-dependent pathway (right) or a Dna2-independent pathway (left) (Reproduced from Henneke et al., 2003a).

2.2.3 Fen1 in DNA repair

Fen1 is involved in base excision repair (BER) as well as in double-strand break repair (DSBR).

BER

BER is the major mechanism for repair of damaged bases in DNA or apurinic/apyrimidinic (AP) sites (reviewed in Hoeijmakers, 2001). Two different pathways can then be followed in higher eukaryotes, the long-patch and the short-patch BER, whereas in mammals the latter is the dominant mode. A requirement for Fen1 in BER was suggested by genetic experiments in *S. cerevisiae* in which RAD27 (homologue of human Fen1) mutants showed high sensitivity to methylmethane sulfonate (MMS), a DNA alkylating agent (Reagan et al., 1995). *In vitro* reconstitution of PCNA-dependent long-patch BER (Pascucci et al., 1999) with human proteins identified the six factors, AP endonuclease, replication factor C (RF-C), PCNA, pol δ, Fen1 and DNA ligase I. The 3' terminus created by AP endonuclease cleavage serves as the target to recruit RF-C, which itself assembles PCNA on DNA, followed by the action of pol δ, Fen1 and DNA ligase I. Fen1 excises the 5'-deoxyribose phosphate group, together with adjacent 3'-nt(s) as part of an oligonucleotide (Prasad et al., 2000). Fen1 can recognise and cleave biologically relevant flap substrates that were assembled into nucleosomes without disrupting histone-DNA interactions (Huggins et al., 2002). This is also important, as its substrates are likely to be assembled into chromatin.

DSBR

When DSBs are detected, a complex cascade of reactions is triggered, aimed at halting the cell-cycle machinery and thereby recruiting repair factors (see also introduction about the '9-1-1 complex' below). After replication, when a second identical copy is available, homologous recombination seems to be preferred; otherwise, cells rely on non-homologous end joining (NHEJ), which is a more error-prone repair mechanism. The DSBs can create a large set of different DNA end configurations (among them also flap structures a preferred substrate for Fen1) which mostly need to be processed by nucleases and pols

before ligation. The difficulty in identifying the processing enzymes comes from the fact that the ends can be processed in many different ways; therefore a genetic knockout of any of the processing components does not block the all NHEJ pathways. Currently, it is assumed that the endonuclease but not exonuclease activity of Fen1 is involved in NHEJ (Wu et al., 1999).

2.3 The heterotrimeric checkpoint complex Rad9, Rad1 and Hus1 (the '9-1-1 complex')

2.3.1 DNA damage checkpoint (reviewed in Melo and Toczyski, 2002)

The DNA damage checkpoint is a signal transduction pathway that blocks cell cycle progression or slows the rate at which S phase proceeds when DNA is damaged. It is a surveillance mechanism and is usually not required for cell cycle events, but enforces their proper order, which is especially critical after acute DNA damage or DNA replication error. Furthermore the checkpoint pathway targets the induction of transcription of repair genes, inducible promotion of repair processes, stabilizes stalled replication forks and executes in the worst case apoptosis, when the integrity of the genome cannot be ensured anymore.

The checkpoint proteins can be divided into four groups. First the phosphatindylinositol 3-kinase (PI3K)-like protein kinases, members of this group are the large protein kinases ATM (Ataxia telangiectasia mutated) and ATR (Ataxia- and Rad-related). They are thought to bind damaged DNA. Either ATM and/or ATR are required for each of the DNA-damage-responsive checkpoints. ATR is constitutively associated with Rad26, which may act as its regulatory subunit. Second the PCNA-like group, which is a complex of the three proteins Rad9, Rad1 and Hus1 thought to form a PCNA-like heteromeric trimer called the '9-1-1 complex' that can be loaded directly at sites of DNA damage. The other two groups contain the two serine/threonine (S/T) kinases – Chk1 and Chk2 – and their adaptors. Chk1 and Chk2 are thought to be activated by ATM/ATR and

phosphorylate targets of the checkpoint. The two adaptors proteins bind the S/T kinases and aid in their activation. If one considers ATM and ATR to act as 'sensor kinases' at the top of the DNA damage checkpoint signalling pathway, the S/T kinases that function downstream in the cascade could be referred to as the 'effector kinases'.

2.3.2 The structure and function of the '9-1-1 complex'

In *S. pompe* six checkpoint genes have been identified called Rad1, Rad3 (ATR), Rad9, Rad17, Rad26 (ATRIP) and Hus1 (al-Khodairy and Carr, 1992; al-Khodairy et al., 1994, Enoch et al., 1992, Rowley et al., 1992)), if no name is bracketed it is the same for the yeast and the human homologue. These genes were found to be involved in G2 checkpoint pathway. When it was shown that many of these genes were conserved from yeast to eukaryotes, it was suggested that yeast G2 checkpoint signalling mechanisms might be similar to that of humans.

Rad9, Rad1 and Hus1 have been shown to interact by immunoprecipitation and by yeast-two-hybrid analysis (Caspari et al., 2000, Hang and Lieberman, 2000, St Onge et al., 1999, Volkmer and Karnitz, 1999). By size-exclusion chromatography it has been shown that they interact in a head-to-tail manner, supporting the model of a circular organisation of these three proteins (Venclovas and Thelen, 2000). Molecular modelling studies were used to propose functions for Rad9, Rad1 and Hus1 (Venclovas and Thelen, 2000, Caspari et al., 2000, Cai et al., 2000). They support the model that Rad9, Rad1 and Hus1 form a heterotrimeric, PCNA-like clamp (*figure 3*). PCNA is a homotrimeric ring that is loaded onto DNA by a clamp loader, RF-C in an ATP-dependent manner (Hubscher et al., 2002, Mossi and Hubscher,1998). Once loaded, PCNA encircles the DNA as a sliding clamp and tethers replication proteins. Studies on the '9-1-1 complex' showed that this complex associates with chromatin after DNA damage (Burtelow et al., 2000). The recruitment of Rad9 to damage sites depends not only on Rad1 and Hus1, but also upon

Rad17 (Melo et al., 2001, Kondo et al., 2001). Rad17 is similar in sequence to the large subunit of RF-C. Mass spectrophotometric analysis of Rad17 complexes in *S. cerevisiae* showed that Rad17 replaces the large subunit and exists in a complex with the other four small RF-C subunits (Green et al., 2000). Several studies showed that the '9-1-1 complex' is loaded onto damaged DNA by the Rad17 – RF-$C_{(2-5)}$ complex (Majka and Burgers, 2003, Ellison and Stillman, 2003, Bermudez et al., 2003). Once loaded, this Rad9-Rad1-Hus1-complex could be a platform to allow other enzymes to repair DNA lesions in a processive manner.

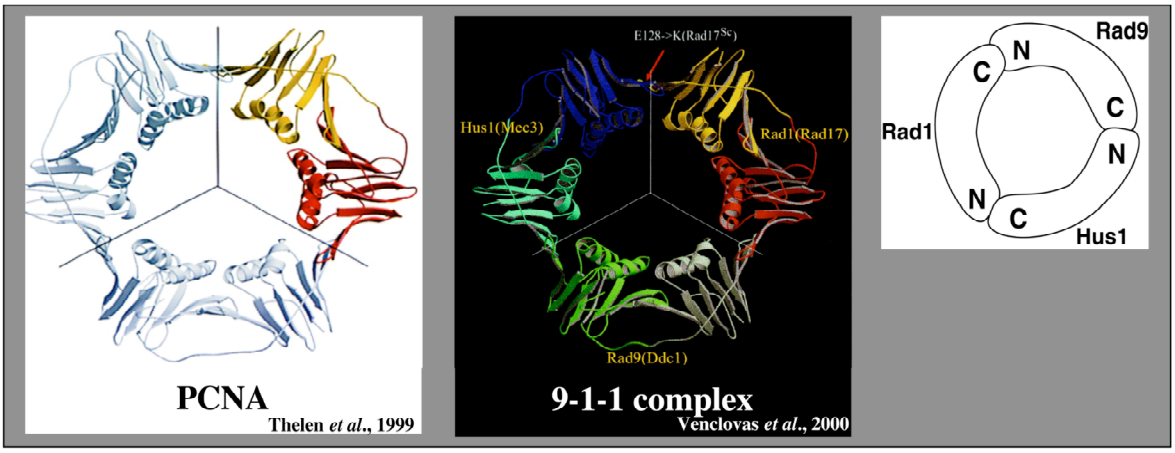

***Figure 3*. Structure comparison of PCNA and the '9-1-1 complex.** 9-1-1 is a heterotrimeric complex: Rad9 (43kDa), Rad1 (31 kDa), Hus1 (32 kDa). The three subunits interact in a head-to-tail manner. Structure prediction and transmission electron microscopy showed a PCNA like ring shape → alternative DNA clamp. (Reproduced from Thelen et al., 1999 and Venclovas et al., 2000)

3 Aim and Project description

The discovery that the three checkpoint proteins – Rad9, Rad1 and Hus1 (the '9-1-1' complex) – share sequence similarity with PCNA has generated many predictions about the structure and function of these proteins (Venclovas and Thelen, 2000). From this structural affinity arose also the idea for this thesis project. PCNA is known to be a functional interaction partner of Fen1. This interaction is well studied in the Hübscher group (Jónsson et al., 1998, Stucki et al., 2001). Direct interaction was shown by pulldown experiment and immunoprecipitation. It was also demonstrated that Fen1 is highly stimulated by PCNA on a single flap as well as on a double flap substrate. This mechanism is notably important in DNA replication. The idea was to test if the three checkpoint proteins Rad9, Rad1 and Hus1 as a complex as well as individually could bind to Fen1 and what could be the functional consequences of such an interaction. The focus during this thesis project was clearly more on the interaction part than on the functional part. The first task was to clarify if the two proteins could interact directly or indirectly, for this pull down assays and Far Western Blots had to be established and optimized. Moreover the behaviour of several Fen1 mutants was tested under different conditions. Finally the impact of Fen1's posttranslational modifications on the Fen1/9-1-1 interaction was studied.

This thesis work was part of the '9-1-1 project' under the supervision of Dr. Magali Toueille with the final goal to learn more on the role of the '9-1-1 complex' in DNA repair.

4 Material and Methods

4.1 Nucleic acid substrates

Oligonucleotides for preparing the substrates for Fen1 nuclease assays were purchased from Microsynth GmbH (Balgach, Switzerland) and their sequences are listed in *table I*. The oligonucleotide Ft2_01 was labelled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase and its recommended reaction buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DDT] from New England Biolabs. The reaction was incubated at 37°C for 45 min and the enzyme inactivated by heating to 80°C for 15 min. Free ATP was removed on Microspin™ G-25 columns. To generate the substrates for the nuclease assays, the appropriate oligonucleotides were mixed in a 1:1 molar ratio in 20mM Tris-HCl (pH 7.4), 150 mM NaCl, heated to 75°C and slowly cooled down to room temperature. The substrates for the electromobility shift assays were also prepared according to this protocol.

***Table I*. Oligonucleotides to create Fen1 substrates used in this study**

Oligonucleotide	Size	Sequence (5′ to 3′)
Upstream primer		
Ft2_02	30-mer	TCGAGGTCGACGGTATCGATAAGCTTGATA
Downstream primer		
Ft2_01	39-mer	**GTCATGATAGATCTGATCGC**TCGAATTCCTGCAGCCCGG
Template		
Ft2_04	49-mer	CCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCG A

Unannealed nt are documented in bold.

4.2 Proteins

4.2.1 Fen1

Human Fen1 cDNA was cloned into the pET 23d vector (Novagen) (Stucki et al., 2001). The ΔP and the ΔC mutants were purified as described previously (Stucki et al., 2001). Wt and mutant proteins were overexpressed in the *E. coli* strain BL21(DE3)pLysS as histidine-tagged proteins. The cells were grown in 1 l of LB medium and induced with 0.5 mM ITPG when OD_{600nm}= 0.5 – 0.7 was reached. After 3 h at 37°C the cultures were centrifuged 20 min at 4,500 rpm using a G6000 rotor (Sorvall© Instruments) and the remaining pellet from 1 l culture was re-suspended in 30 ml buffer A [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 1 mM PMSF] and sonicated (Branson Sonifier cell disruptor 1315) [duty cycle 60°, 2 x 15-25 pulses]. After centrifugation at 20,000 rpm using a SS34 rotor (RC5C, Sorvall© Instruments) for 30 min at 4 °C the supernatant was filtered (FP 30/0,45 CA-S, Schleicher&Schuell) and loaded on a Nickel charged metal chelating resin (HiTrap, Amersham Pharmacia Biotech). For this and all subsequent purification steps fast protein liquid chromatography (FPLC, Amersham Pharmacia Biotech) was used. Before and after loading the column was washed with buffer B [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole]. Bound proteins were eluted with a linear imidazole gradient from 20 to 500 mM. The fractions were analyzed on Coomassie stained SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) and pooled according to this. The pool of the peak-fractions was diluted 1:10 in buffer C [10 mM Tris-HCl (pH 7.6), 1 mM DTT, 10% [v/v] glycerol] and injected to a SP-Sepharose® column (Amersham Pharmacia Biotech). Bound proteins were eluted with a linear NaCl gradient from 0.03 to 1.0 M in the same buffer. The fractions were analyzed by SDS-PAGE and those showing most homogeneity were pooled and dialyzed into storage buffer [20 mM Tris-HCl (pH 7.6), 0.01% [v/v] NP-40, 0.5 mM EDTA, 1 mM DTT, 60% [v/v] glycerol] using dialysis cassettes (Slide-A-Lyzer®10K, Pierce).

4.2.2 '9-1-1 complex'

The '9-1-1 complex' was isolated by co-expressing the three baculovirus encoding the recombinant hRad9, hRad1 and hHus1 in Sf9 cells (provided by T. Tsurimoto, Japan). A protocol slightly modified from the one published by Shiomi et al., 2002 was used. 72 h after infection, $1x10^8$ cells were lysed with 0.5% [v/v] NP-40 in 10 ml buffer B [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10% [v/v] glycerol, 10 mM DTT, 1 mM PMSF, 20 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml bestatin] at 0 °C for 30 min and centrifuged at 20,000 rpm using a SS34 rotor (RC5C, Sorvall© Instruments) for 2 h at 4 °C. The resulting lysate was either used as '9-1-1 extracts' for PD experiments or loaded on to a DEAE Sepharose® column (Amersham Pharmacia Biotech) equilibrated with buffer H [25 mM HEPES-NaOH (pH 8.1), 1 mM EDTA, 10% [v/v] glycerol, 0.01% [v/v] NP-40, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml bestatin] containing 0.1 M NaCl. After washing the column with 0.1 M NaCl, bound proteins were eluted with buffer H containing 0.4 M NaCl. Rad9-, Hus1- and Rad1-containing fractions were determined by monitoring the UV absorption. At this stage, Rad9, Hus1 and Rad1 were the major components of the fractions. Pooled fractions were diluted 1:4 in buffer H containing 0.1 M NaCl and loaded on to a Q-Sepharose® column (Amersham Pharmacia Biotech). Bound proteins were eluted with a linear NaCl gradient from 0.1 to 0.6 M in the same buffer. Fractions containing Rad9, Hus1 and Rad1 were pooled according to the result obtained by Western Blot analysis. This pool was called '9-1-1' QS. The pool was dialyzed in 9-1-1 storage buffer [25 mM HEPES (pH 8.1), 1 mM EDTA, 20% [v/v] glycerol, 100 mM NaCl, 1 mM β-mercaptoethanol, 0.1 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml pepstatin, 2 µg/ml bestatin] using dialysis cassettes (Slide-A-Lyzer®10K, Pierce).

According the same protocol as for the '9-1-1 extract' extracts from Sf9 cells overexpressing only one of the three subunits either Rad9, Rad1 or Hus1

were obtained. For negative controls extract from mock-infected Sf9 cells was used.

4.2.3 PCNA

Human PCNA was produced in *E. coli* strain BL21(DE3)pLysS using the plasmid pT7/hPCNA (Schurtenberger et al., 1998). PCNA was isolated from 1 l culture in LB medium. The cells were induced with 0.5 mM ITPG when OD_{600nm}= 0.8 was reached. After 4 h at 37°C the cultures were centrifuged 20 min at 4,500 rpm using a G6000 rotor (Sorvall© Instruments) and the pellet was re-suspended in 20 ml buffer A [20 mM Tris-HCl (pH 6.8), 25 mM NaCl, 1 mM EDTA, 0.01% [v/v] NP-40, 1 mM DTT, 10 mM $NaHSO_3$, 1 mM PMSF] and sonicated (Branson Sonifier cell disruptor 1315) [duty cycle 60°, 15-25 pulses]. After centrifugation at 20,000 rpm using a SS34 rota rotor (RC5C, Sorvall© Instruments) for 30 min at 4 °C the supernatant was loaded on a cellulose phosphate P11 column (Whatman) connected to a Q-Sepharose® Fast Flow column (Amersham Pharmacia Biotech). Both columns were equilibrated in buffer QS [20 mM Tris-HCl (pH 6.8), 1 mM EDTA, 10 mM $NaHSO_3$, 0.01% [v/v] NP-40, 10% [v/v] glycerol]. The columns were washed twice with buffer QS with additional 100 mM NaCl, plus twice only the Q-Sepharose® Fast Flow column was washed. Bound proteins were eluted with a linear NaCl gradient from 0.1 to 0.75 M. The fractions were analyzed on Coomassie stained SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva) and pooled according to this. For all further purification steps FPLC (Amersham Pharmacia Biotech) was used. The pool of the peak-fractions was adjusted to 1 mM $CaCl_2$ and injected to a Econo-Pac® CHT-II (hydroxyapatite) column (Bio-Rad Labotatories) previously equilibrated with CHT I buffer [10 mM Tris-HCl (pH 6.8), 300 mM NaCl, 5% [v/v] glycerol]. The column was washed twice, once with CHT II buffer [10 mM Tris-HCl (pH 6.8), 1 mM $MgCl_2$, 5% [v/v] glycerol] and once with CHT III buffer [10 mM Tris-HCl (pH 6.8), 50 mM NaCl, 5% [v/v] glycerol]. Bound proteins were eluted with a linear $NaPO_4$ gradient from 0.0 to 0.25 M in the same buffer. The fractions were

analyzed by SDS-PAGE and those showing most homogeneity were pooled and dialyzed in storage buffer [25 mM Tris-HCl (pH 7.5), 50% [v/v] glycerol, 50 mM NaCl, 1mM EDTA, 1 mM DTT].

4.2.4 GroEL

GroEL (only apical domain) was kindly provided by Erica Friedrich-Heineken and purified as described in her diploma work, (Friedrich-Heineken E., "Refolding of a small subunit of RF-C using immobilized GroEL mini-chaperones", Diploma University of Zurich, 2000).

4.2.5 GST-p300-HAT

The GST-tagged HAT domain of p300 was kindly provided by Christine Bürki (this Institute).

4.2.6 GST-p300 #4

The GST-tagged fragment number 4 (#4) (aa 1'459-1'892) of p300 was kindly provided by Michael Hottigers group (this Institute). It was described in Hasan et al., 2001.

4.3 Fen1 nuclease assay

The assay conditions were identical to those published in Friedrich-Heineken et al., 2003. The enzyme-titration assays were performed in a final volume of 12.5 μl containing the following ingredients: 40 mM Tris-HCl (pH 7.5) or 50 mM Bis-Tris (pH 6.5), 10 mM $MgCl_2$, 1 mM DTT, 200 μg/ml BSA and 50 fmol of DNA substrate. After addition of Fen1 wt, reactions were incubated for 15 min at 30°C and stopped with a 2.5x stop buffer (95% [v/v] formamid, 20 mM

EDTA, 0.05% [w/v] each bromophenol blue and xylene cyanol). The remaining substrates were denatured by heating to 95°C for 4 min. Products were separated on 15% denaturing polyacrylamide (Acrylamide/Bis-acrylamide, 19:1 mixture, 40% [w/v] (Qbiogene)) gels containing 7 M urea (BioRad gel apparatus) and visualised by autoradiography. The X-ray film (Contatyp, Typon Imaging AG) was exposed for 2 h at –80°C using a highly sensitive screen (Okamato). For PCNA stimulation of Fen1, the reactions were performed as described above except that 100 mM NaCl was added to the buffer, this allows the detection of Fen1 stimulation by PCNA (Jónsson et al., 1998). For the influence of the '9-1-1 complex' conditions with 0 mM and 100 mM NaCl were tested.

4.4 Electrophoresis mobility shift assay (EMSA) for Fen1

Assay conditions were the same as published in Friedrich-Heineken et al., 2003. The binding reactions were carried out in a final volume of 20 µl containing: 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 5 mM EDTA, 100 µg BSA, 4% [w/v] Ficoll, 50 fmol of labelled substrate and the indicated amounts of Fen1 wt and PCNA, respectively '9-1-1 complex'. After incubation for 10 min at room temperature, reactions were loaded on 6% polyacrylamide (Acrylamide/Bis-acrylamide, 19:1 mixture, 40% [w/v] (Qbiogene)) gels containing 0.5x TBE and run first at 50 V for 45 min and then at 100 V for 90 min (BioRad gel apparatus). Finally, the bands were visualised by autoradiography. The X-ray film (Contatyp, Typon Imaging AG) was exposed over night at –80°C using a highly sensitive screen (Okamato).

4.5 Native Gel

The electrophoresis was performed in 4-20% native polyacrylamide gels using the Novex® Tris-Glycine Gels (Invitrogen) according to the manufacturer's instructions. The running buffer [25 mM Tris base, 192 mM glycine, pH 8.3] and

the sample buffer [100 mM Tris-HCl, 10% [v/v] glycerol, 0.0025% [w/v] bromphenol blue, pH 8.6] for native gels were also purchased from Invitrogen. The products on the gel were analysed by Western Blot. The transfer buffer had additional 0.005% [w/v] SDS included; the gel was shortly pre-incubated in transfer buffer before blotting.

4.6 In vitro acetylation assay

Fen1 was *in vitro* acetylated as described before (Hasan et al., 2001). 1 and 4 µg each of purified Fen1 wt or its mutants were incubated with 50 µM [^{14}C] acetyl coenzyme A [0.05 mCi/ml] (MG 269, Moravek Biochemicals) in 30 µl HAT buffer [50 mM Tris-HCl (pH 8.0), 10% [v/v] glycerol, 80 mM NaCl, 1 mM DTT, 1 mM PMSF, 10 mM Na-butyrate] at 30°C for 1 h together with 1 µg of purified GST-p300-HAT. 10x SDS loading buffer was added, the reactions were heated to 95°C for 4 min and the samples were subjected to a 10 % SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) analysis (BioRad gel apparatus). Gels were stained with Coomassie blue. Before vacuum drying (Gel Dryer Model 583, BioRad) the signal was enhanced with 1 M sodiumsalicylat and the bands were visualised by over night exposure to an x-ray film (Contatyp, Typon Imaging AG).

4.7 Binding assay

Different amounts of Fen1 wt or mutant proteins were bound to 50 µl 50% [v/v] Ni^{2+}-beads (ProBond^{TM} Nickel-Chelating Resin, Invitrogen) in 300 µl PD buffer [50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 0.025% [v/v] NP-40]. The reactions were incubated 1 h at 4°C with constant gentle agitation. The beads were then washed 5 times with 1 ml PD buffer but in addition 10 mM imidazole was included. Bound proteins were eluted by boiling in a 2x SDS-PAGE loading

buffer and resolved on a 12% SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) gel which was subsequently stained in Coomassie blue.

4.8 Pull down assay with $(his)_6$-Fen1

Each step of the PD assay was performed in a final volume of 350 µl. 8 µg of Fen1 wt or mutant proteins in 300 µl PD buffer [50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 0.025% [v/v] NP-40] were bound on 50 µl 50% [v/v] Ni^{2+}-beads (ProBond™ Nickel-Chelating Resin, Invitrogen). The reactions were incubated 1 h at 4°C with constant gentle agitation. After centrifuging 2 min at 2000 rpm (Centrifuge 5417R, Eppendorf) the supernatant was thrown away and the beads were washed 4 times with 1 ml PD buffer. To prevent unspecific binding, the beads were pretreated with 10 µg of Sf9 cell extract (2 h incubation at 4°C) and washed as described above. In a next step the indicated amount of '9-1-1 complex', Sf9 extract, PCNA or p300 were added and again incubated for 2 h at 4°C. The beads were then washed 5 times with 1 ml PD buffer but in addition 10 mM imidazole was included. Bound proteins were eluted by boiling in a 2x SDS-PAGE loading buffer and resolved on a 12% SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) gel. The proteins bound to Fen1 were detected by Western Blot analysis.

For negative controls reactions were performed with GroEL as described above except that instead of Fen1 10 µg of GroEL was bound to the beads.

The pull down competition assay was carried out following the same protocol. For the third incubation step the '9-1-1 complex' and its competitor protein either PCNA or p300 #4 were added simultaneously, at different ratios (see Results).

4.9 Pull down assay with in vitro acetylated $(his)_6$-Fen1

12 µg of purified his-tagged Fen1 wt were *in vitro* acetylated as described above using 50 µM unlabled acetyl coenzyme A (Sigma). After 1 h incubation at 30°C the reaction mix was directly added to 50 µl 50% [v/v] Ni^{2+}-beads (ProBond™ Nickel-Chelating Resin, Invitrogen) in 300 µl PD buffer [50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 0.025% [v/v] NP-40]. Fen1 was bound to Ni^{2+}-beads during 1 h at 4°C with constant gentle agitation. The beads were washed four times, twice with 1 ml PD buffer including 500 mM NaCl and twice with PD buffer (80 mM NaCl). All further steps were performed as described for PD assay.

4.10 Western Blot

Proteins separated by SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) were electroblotted during 2 h at 100 V (BioRad Western Blot Apparatus) onto nitrocellulose membrane (Osmonics). The transfer buffer [25 mM Tris-Base, 192 mM glycine, 20% [v/v] methanol] had a temperature of 4°C and was cooled during blotting with ice. After staining 5 min in Ponceau solution the membrane was cut horizontally in two parts if needed and blocked with TBST and 5% [w/v] powdered milk. The appropriate antibodies were diluted in TBST with 2% [w/v] powdered milk. The α Rad9 and the α Hus1 antibodies were kindly provided by Raimundo Freire, Tenerife (Spain). They were produced in rabbits and the serums are used in a 1:10,000 dilution. The monoclonal mouse-α PCNA (PC10), used diluted 1:2,000, the polyclonal goat-α Rad1 (N-18), used diluted 1:500, and the polyclonal rabbit-α GST (Z-5), used diluted 1:5,000, antibodies were purchased from Santa Cruz Biotechnology, California. The membranes were incubated with the primary antibody either 3 h at room temperature or over night at 4°C and then 3 times 5 min washed with TBST. Finally the corresponding HRP-conjugated secondary antibody (donkey anti-goat IgG diluted 1:2,500 (Santa Cruz Biotechnology), anti-rabbit Ig diluted 1:5'000 and anti-mouse Ig

1:5,000 (Amersham Pharmacia Biotech)) in blocking solution, was added, the membrane incubated for 1 h at room temperature, followed by 3 times 5 min washings with TBST (0.05% [v/v] Tween 20) and 10 min with TBS. Antibodies bound to the proteins were detected by Uptilight HRP Blot Chemiluminescent Substrate (Uptima) followed by exposure to an X-ray film (Contatyp, Typon Imaging AG).

4.11 Far Western Analysis

According to the protocol published in (Wu et al., 2000). Increasing amounts (0.5 µg, 0.75 µg, 1µg) were dissolved on a 12% SDS-PAGE (Acrylamide/Bis, solution (37.5:1), 40% [w/v] (Serva)) and then electroblotted during 1.5 h at 100 V (BioRad Western Blot Apparatus) onto nitrocellulose membrane (Osmonics). The transfere buffer [25 mM Tris-Base, 192 mM glycine, 20% [v/v] methanol] had a temperature of 4°C and was cooled during blotting with ice. After staining 5 min in Ponceau solution the membrane was cut vertically along the lanes. The membranes were immersed twice in denaturation buffer [6 M guanidine-HCl in PBS] for 10 min and then incubated six times for 10 min in serial dilutions (1:1) of denaturation buffer supplemented with 1 mM DTT. Afterwards they were blocked for 30 min at 4°C with TBST (containing 0.3% [v/v] Tween 20) and 10% [w/v] powdered milk before being incubated 1 h at 4°C with Rad9, Rad1 or Hus1, two dilutions of each were used 50 respectively 100 µg/ml. The proteins were diluted in TBST (containing 0.3% [v/v] Tween 20) supplemented with 0.25% [w/v] powdered milk, 1 mM DTT and 1 mM PMSF. The membranes were washed four times for 10 min each in TBST (containing 0.3% [v/v] Tween 20) with 0.25% [w/v] powdered milk. The second wash contained 0.001% [v/v] glutaraldehyde. Conventional Western Blot analysis was then performed as described above to detect Rad9, Rad1 and Hus1 bound to Fen1.

5 Results

5.1 Rad9, Rad1 and Hus1 form a heterotrimeric complex

The three checkpoint proteins Rad9, Rad1 and Hus1 were purified from Sf9 cells infected with baculovirus (see Materials and Methods). With Western Blot analysis it could easily been demonstrated, that if the '9-1-1 complex' is purified from triple infected Sf9 cell extract, the three subunits are co-eluted (*figure 4a*), but the results did not give any information about complex formation among the three proteins. To answer this question samples of extracts from single and triple infected Sf9 cells were resolved on native gradient gels and analyzed by Western Blot (*figure 4b*). In the lanes were the '9-1-1 extract' was loaded, bands for Rad9, Rad1 and Hus1 appeared at the same position. This suggested that in extract from triple infected Sf9 cells, Rad9, Rad1 and Hus1 form a complex. For all fractions positive signals could also immunologically be detected at the position of the subunits, because not all monomers are integrated in a complex.

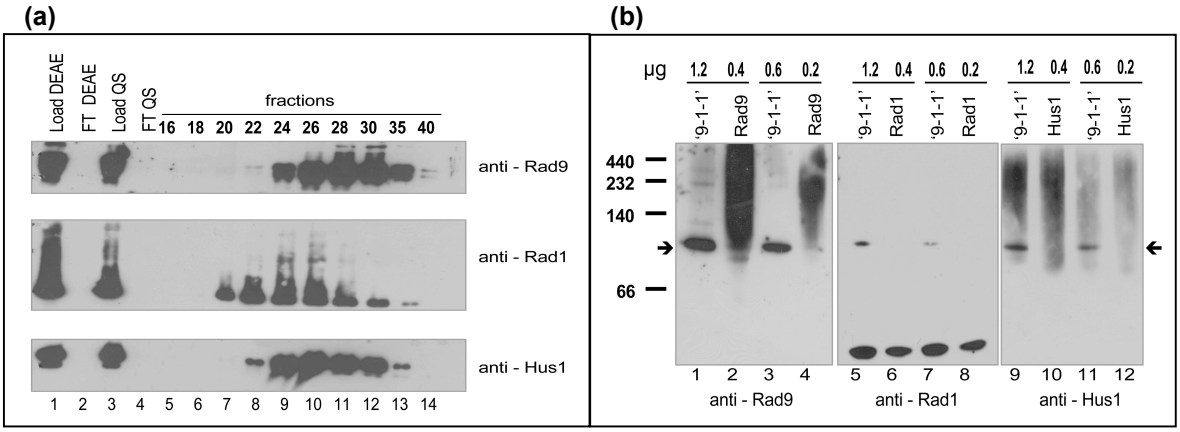

Figure 4. **(a) Purification of the '9-1-1' complex.** The '9-1-1 complex' fraction QS was purified over two columns, first a DEAE Sepharose and second a Q-Sepharose. In lane 1, respectively 3, samples of the loads were resolved, lanes 2 and 4 shows the corresponding flow through (FT) fractions. The complex was purified with FPLC system and after the Q-Sepharose column fractions of 250 µl were collected and analyzed by WB. The three panels show the membranes developed with the three antibodies αRad9, αRad1 and αHus1. The three subunits were co-eluted in fractions 25 to 35, which were pooled to the '9-1-1' QS fraction. **(b) Native gradient gel.** Samples of extract from single and triple infected Sf9 cells were analyzed by WB from a native gradient gel. The amount of the extract from triple infected cells is three times the amount of extract form single infected cells. Two different amounts of each extract were loaded. At the position of about 110 kDa (marked with an arrow) a band appears on all three panels (αRad9, αRad1 and αHus1) in the lanes were extract from triple infected cells were loaded. On the panel in the middle (αRad1) it is obvious that together with the complex (position of the arrow) also free subunits are co-existing visible (at the bottom of the gel).

5.2 The purified '9-1-1 complex' contains a nuclease activity

From previous studies it is evident that the biochemical characteristics of Fen1 are well known (reviewed in Henneke et al., 2003a). Among these data it was also shown that PCNA is one of the important interaction partner of Fen1 and that the Fen1 activity is stimulated by PCNA (Shen et al., 1998, Stucki et al., 2001, Warbrick et al., 1997). PCNA on the other hand has sequential and structural similarities to the checkpoint protein complex Rad9, Rad1 and Hus1 (called '9-1-1 complex') (Venclovas and Thelen, 2000), this gave the idea to perform experiments with Fen1 and the '9-1-1 complex' in order to test the effect of the latter on the Fen1 activity and compare it to the stimulatory effect of PCNA. In a first set of experiments various parameters (pH, NaCl concentration) were

tested in a Fen1 single flap endonuclease assay (see Materials and Methods). The published results for the stimulatory effect of PCNA on Fen1 could be reproduced *(figure 5a).*

For the '9-1-1 complex' in yeast, the Ddc1, Mec3 and Rad17 complex, it was shown that these three proteins as a complex have no exonuclease activity (Giannattasio et al., 2004), an exonuclease activity was only claimed for human Rad9 alone (Bessho and Sancar, 2000). However in a Fen1 activity assay the '9-1-1 complex' alone seems to cleave the substrate unspecifically (*figure 5*). One main product could be observed with an estimated length of about one nt (no marker were used in this experiment). Additional 100 mM NaCl apparently decreases this nuclease activity to about 30 %, whereas at pH 7.5 the efficiency is only slightly better than at pH 6.5. The '9-1-1 complex' seems to have an inhibitory effect on the Fen1 activity, but the result could also be interpreted as competition between the two nuclease activities for the same substrate and so this did not allow drawing any conclusions about the effect of the '9-1-1 complex' on Fen1. About the nature of this additional nuclease only speculation is possible, it looks like it is a 5' → 3' exonuclease. Data from the literature suggested that this nuclease activity does not belong to the '9-1-1 complex' but rather to a co-purified nuclease. The purification of 'nuclease-free 9-1-1 complex' was repeated. The data obtained confirm the hypothesis of a co-purified nuclease. To remove this nuclease appears to be very tricky. Further studies are required to solve this problem.

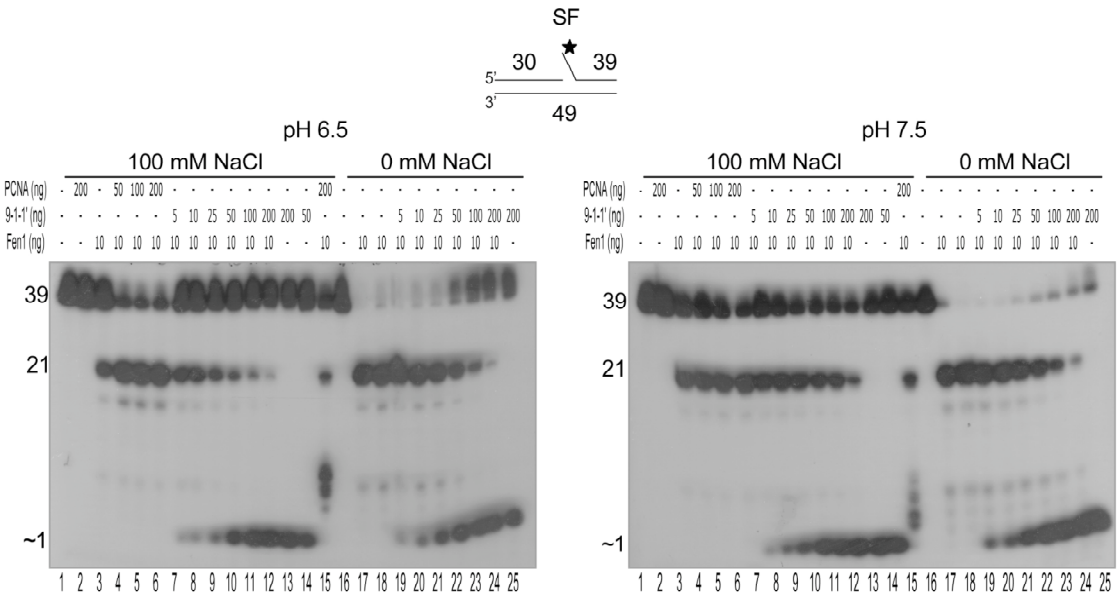

***Figure 5*. PCNA and the '9-1-1 complex' influence Fen1 activity differently.** A single flap (SF) substrate as drawn was used for the Fen1 activity assays. The optimal conditions for PCNA stimulation are known to be at pH 6.5 with 100 mM NaCl (left panel lane 1 to 6). The NaCl is required to inhibit Fen1 and to make the stimulation possible. When the '9-1-1 complex' was added to the reaction a decrease of the Fen1 product (21 nt) could be observed, but at the same time an additional product increased at the position of about 1 nt. The effect occured at pH 6.5 as well as at pH 7.5. NaCl seems to have only a slight inhibitory influence. The additional product was probably cleaved by a co-purified exonuclease. In lane 18 the volume of the '9-1-1 complex' was substituted by '9-1-1' storage buffer. Lane 15, PCNA from an other purification was tested.

PCNA can also stimulate Fen1 binding to DNA (Tom et al., 2000). Next this effect was studied by electro mobility shifting assays (EMSA) (see Materials and Methods). Again the goal was to compare PCNA and the '9-1-1 complex'. The results published by Bambara's group could not be reproduced but an effect of the '9-1-1 complex' could be observed. For EMSA the partially purified QS fraction of the '9-1-1 complex' was used (see Materials and Methods). In an EMSA the '9-1-1 complex' alone without Fen1 gave a shift. With 20 ng of Fen1 and 1 µg of '9-1-1 complex' two bands appeared. This might be either a Fen1-DNA or a lighter '9-1-1'-DNA complex (*figure 6*). This result raised the question whether the '9-1-1 complex can bind to DNA even without the clamp loader

RF-C/Rad 17 or if the effect was due to the co-purified putative nuclease, which could also be responsible for the second band in the last lane. To answer this questions again ‘nuclease free 9-1-1 complex’ is required.

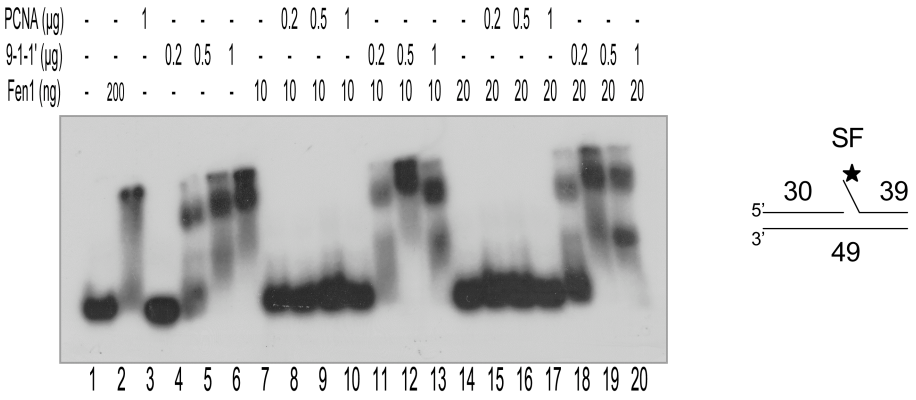

Figure 6. **The ‘9-1-1 complex’ can bind to single flap (SF) DNA.** An Electro mobility shift assay (EMSA) was done as described in ‘Materials and Methodes’. Lane 1: no enzyme control, single flap (SF) substrate; lane 2: positive control, an excess of Fen1 was added to see the position of the Fen1-shift. In lanes 4 – 6 ‘9-1-1 complex’ and the SF substrate were incubated. The ‘9-1-1 complex’ alone provoked a shift at the same position as 10 or 20 ng of Fen1. With the same amounts of PCNA as ‘9-1-1 complex’ no shift appears (lanes 8 – 10 and 15 – 17).)

5.3 Fen1 wt interacts with the ‘9-1-1 complex’ and with each of the three subunits Rad9, Rad1 and Hus1

Next the focus was to characterize the interaction between Fen1 and the ‘9-1-1 complex’. The first task was to optimize the conditions for a Ni^{2+}-pulldown (PD) experiment (see Materials and Methods) with purified His-tagged Fen1 and extracts from Sf9 cells that were triple infected with Rad9, Rad1 and Hus1. The optimal buffer conditions were 80 mM NaCl, 0.025% NP-40. In a binding assay (see Materials and Methods) different amounts of purified proteins were bound to Ni^{2+}-beads. They were: first Fen1 wt (Harrington and Lieber, 1994a); second Fen1 ΔC, a mutant lacking the last 21 aa (aa 360-380 lacking) at the C terminus (Stucki et al., 2001); third Fen1 ΔP, a mutant lacking the PCNA interaction motif

(aa 337-344 lacking) (Stucki et al., 2001); fourth Fen1 D86A, an active site mutant which has a single aa replaced (Stucki M. and Hübscher U., unpublished data) and finally fifth His-tagged GroEL, the apical domain (aa 191-376) of a bacterial chaperon protein, which was used as a negative control. The binding assay gave information about the necessary amount of proteins to cover the beads (*figure 7*).

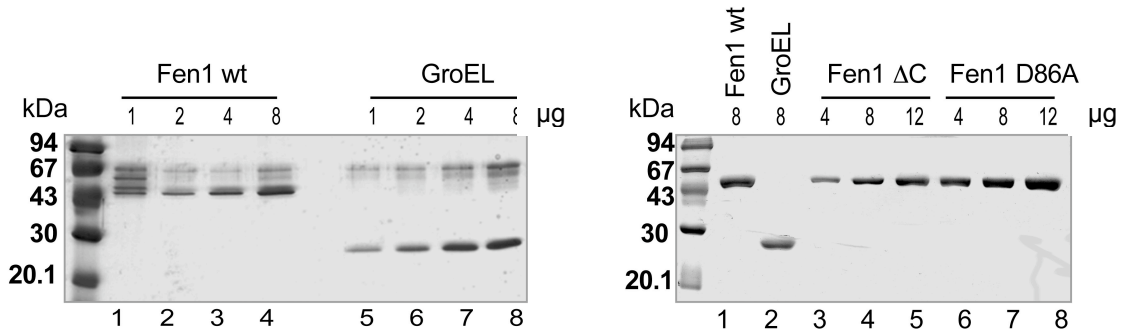

Figure 7. **Optimisation of the Ni^{2+}-beads binding assay.** On the left panel different amounts of Fen1 wt and GroEL were bound to Ni^{2+}-beads. The beads were washed and the elution resolved on a 12% SDS-PAGE. For PD experiments 8 ng of each were bound to 25 µl dry Ni^{2+}-beads. On the right panel 8 ng of Fen1 wt and GroEL were bound to beads as reference values, to which the different amounts of Fen1 ΔC and Fen1 D86A were compared. In PD experiments 15 ng of Fen1 ΔC and 8 ng of Fen1 D86A were bound to the beads. For Fen1 ΔP the same amounts as for Fen1 ΔC were chosen because they have the same binding capacity for Ni^{2+}-beads (data not shown).

At the beginning problems with unspecific binding of the '9-1-1 complex' proteins to the Ni^{2+}-beads and to GroEL were evident. This could be avoided by an additional step during the PD experiment. After the His-tagged proteins were bound to the Ni^{2+}-beads, the beads were washed with PD buffer and then incubated with the same amount of extract from mock infected Sf9 cells as in the third incubation step where extracts of infected Sf9 cells were used. Under optimal conditions (see Materials and Methods) Fen1 wt bound to Ni^{2+}-beads interacted with the '9-1-1 complex' from Sf9 cell extract as well as with the '9-1-1' QS (*figure 8c*). To test the strength of this interaction the experiments were repeated under more stringent conditions by increasing the NaCl and the NP-40

concentrations in the washing buffer (see Materials and Methods). The signal appearing on the film after developing the WB analyze was as intense as before even when a washing buffer containing concentrations of 150 mM NaCl and 0.1% NP-40 was used (*figure 9*). This suggested that Fen1 and the '9-1-1 complex' are interacting very strongly. The PD experiment was analyzed by Western Blot (WB) (see Materials and Methods) which were developed with antibodies (AB) against Rad9, Rad1 and Hus1. From these results it was concluded that Fen1 wt and the '9-1-1 complex' interacted directly.

Next it was tested which of the three subunits of the '9-1-1 complex' is responsible for this interaction. A PD experiment under the same conditions as described before was executed where extracts from singly infected Sf9 cells containing Rad9, Rad1 and Hus1 respectively were tested as interaction partners of Fen1 wt. Surprisingly all three subunits gave a positive signal when the PD assay was analyzed by WB *(figure 8a).* These findings were confirmed by a Far Western blot analysis (see Materials and Methods) (*figure 8b).*

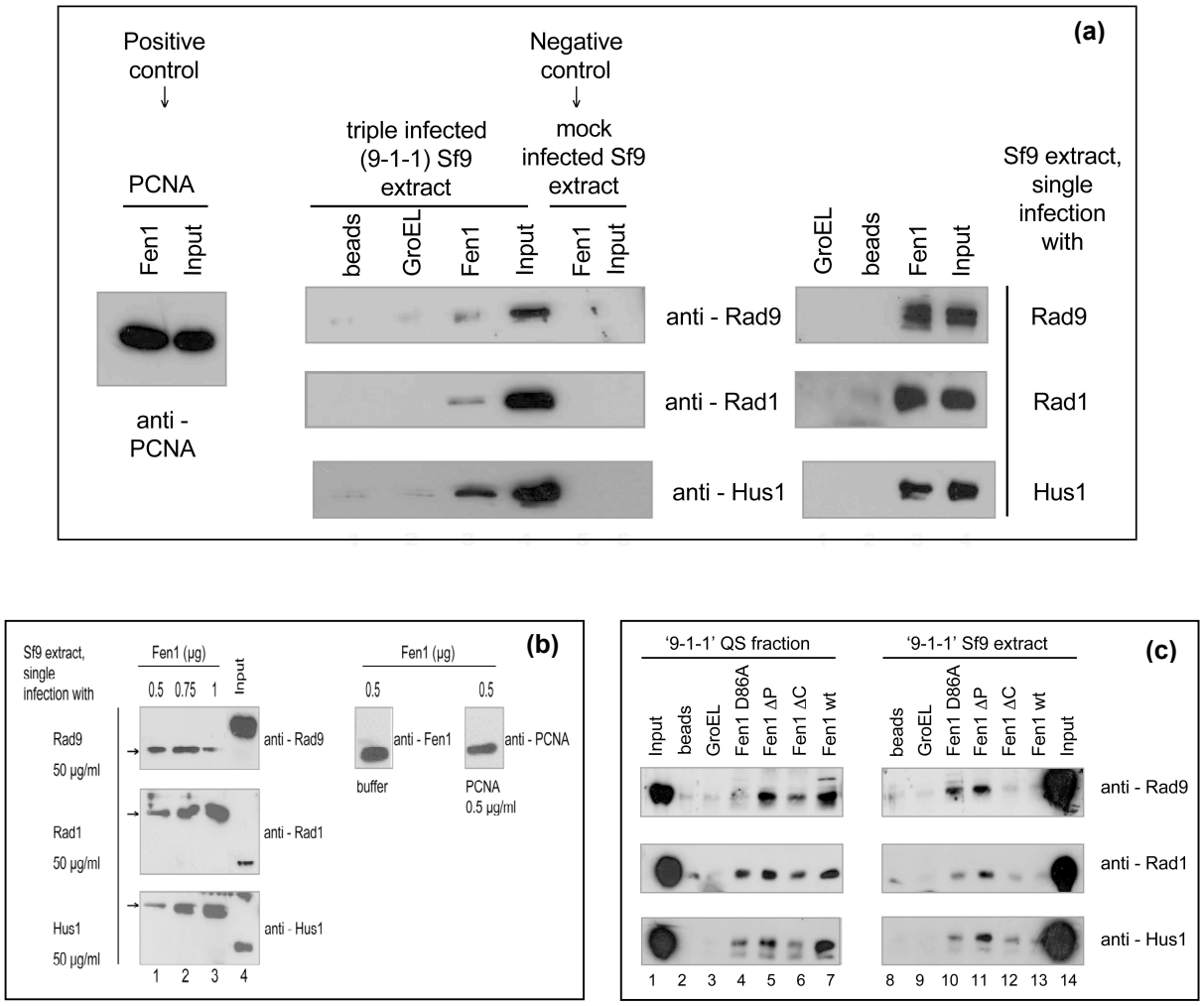

Figure 8. **Fen1 interacts with the '9-1-1 complex' as well as with the three individual subunits Rad9, Rad1 and Hus1. (a)** On the left panel is the positive control of the PD assays, the interaction of Fen1 wt with PCNA gives a positive signal in the WB developed with αPCNA antibody. As a negative control the experiment was performed with Fen1 wt and extract from mock infected Sf9 cells. More controls were done with beads only or GroEL bound to beads, which were incubated with '9-1-1' extract. The results were analyzed by WB using antibodies against Rad9, Rad1 and Hus1. Finally when the interaction of Fen1 wt with '9-1-1' extract was tested, a signal appears for all three antibodies. The same result was obtained when Fen1 wt was incubated individually with extract from each of the subunits Rad9, Rad1 and Hus1. **(b)** The interaction with the three subunits individually was confirmed by a Far Western experiment. Different amounts of Fen1 wt were loaded on a gel and blotted onto a nitrocellulose membrane, which was subsequently incubated with dilutions of extract from single infected cells. Finally a WB using antibodies against Rad9, Rad1 and Hus1 was performed. An arrow on all three panels mark the position of Fen1, the signal is due to the '9-1-1' antibodies. As a positive control PCNA was used. **(c)** Binding of the '9-1-1' QS and '9-1-1' extract to Fen1 was compared. In a PD assay they showed about the same binding capacity. Simultaneously the interaction of the '9-1-1 complex' with several Fen1 mutants was tested. The binding to Fen1 ΔC gives a weaker signal (lanes 6 and 12).

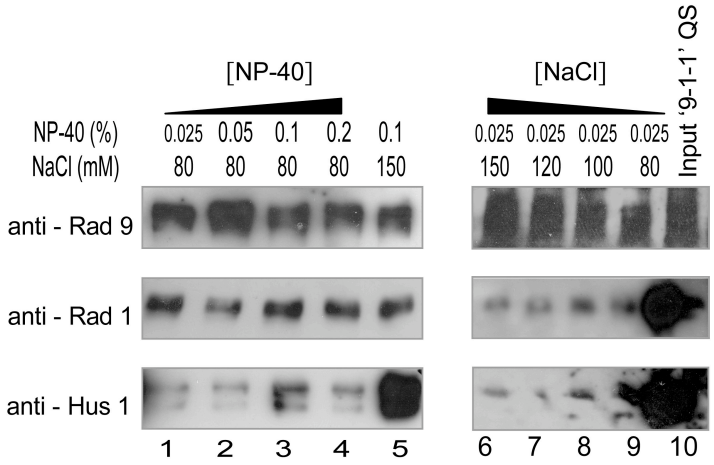

Figure 9. **Fen1 interacts with the '9-1-1 complex' also under more stringent conditions.** Fen1 wt was bound to Ni^{2+}-beads and then incubated with '9-1-1' QS. Washing buffers with increased concentrations of NP-40 and NaCl were tested. Higher NP-40 and/or NaCl concentrations do not prevent the Fen1/9-1-1 interaction.

5.4 Fen1 likely interacts with the '9-1-1 complex' through its C-terminus

The three Fen1 mutants described above, Fen1 ΔC, Fen1 ΔP and Fen1 D86A, were tested in the Fen1/'9-1-1 complex' interaction study hoping to get a first hint where on Fen1 the binding site for the '9-1-1 complex' is located. They were tested in a PD assay under optimal and also more stringent conditions and compared to Fen1 wt binding to the'9-1-1 complex' (*figure 9*). Binding of the '9-1-1 complex' to Fen1 ΔC was weaker than to Fen1 wt. Between Fen1 wt and ΔP no difference in the binding capacity could be observed (*figure 8c)*. The active site mutant of Fen1 interacted only slightly weaker with the '9-1-1 complex' than the wild type, this mutant can therefore be used as a negative control for further functional studies with Fen1 and the '9-1-1 complex', in which the activity of Fen1 is not wanted.

These data suggested that PCNA and the '9-1-1 complex' don't bind to the same region of Fen1 and that the C-terminus of Fen1 might be important for the interaction with the '9-1-1 complex'. When the interaction of Fen1 ΔC with the

three monomers of the '9-1-1 complex' Rad9, Rad1 and Hus1 was tested it turned out that Rad9 was strongly interacting, whereas Rad1 and Hus1 were hardly binding to Fen1 ΔC *(figure 10).*

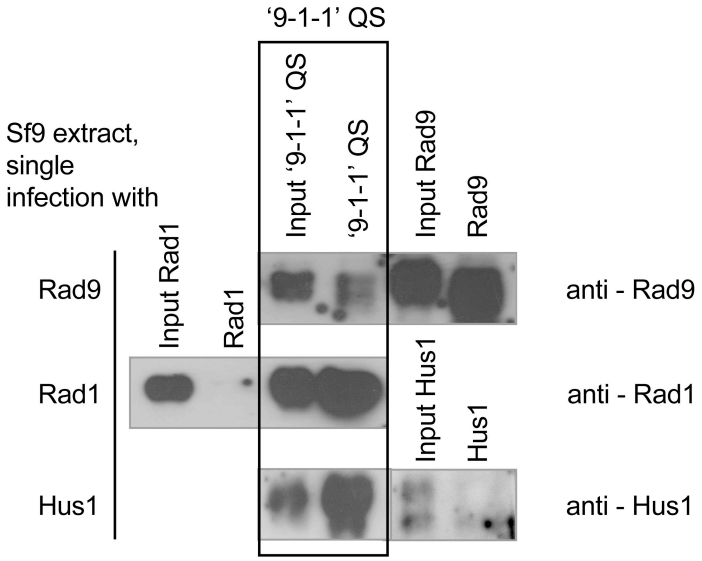

Figure 10. **Interaction of Fen1 ΔC with the three subunits of the '9-1-1 complex'.** A PD assay with Fen1 ΔC and extract from single infected Sf9 cells was performed. The two lanes in the middle show the input of '9-1-1' QS and the interaction of Fen1 ΔC and the '9-1-1' QS. Fen1 ΔC binds to the Rad9 subunits (upper panel on the right) but hardly to Rad1 (middle panel on the left) and Hus1 (bottom panel on the right).

To support this hypothesis PD competition assays were performed. First, Fen1 was bound to Ni^{2+}-beads then increasing amounts of two competitor proteins were added and incubated simultaneously. PCNA and the partially purified '9-1-1 complex' QS fraction were tested as competitors in a first experiment. 1.7 µg of '9-1-1 complex' was compared to 0.46 µg of PCNA; this is a molar ratio PCNA to '9-1-1 complex' of 1:3.4. PCNA was increased to 0.92 µg and 1.84 µg, '9-1-1 complex' was kept at a constant amount. Vice versa a constant amount of 0.46 µg of PCNA was compared to 3.4 µg and 6.8 µg of '9-1-

1 complex'. The PD assay was analyzed by WB, and the data indicated that as expected no competition could be observed (*figure 11a*). These data supported the assumption that the PCNA binding site of Fen1 is likely not involved in the interaction between the '9-1-1 complex' and Fen1.

Finally this experiment was repeated but PCNA was replaced by the transcriptional coactivator p300 fragment #4 (overexpressed in *E. coli*), which is known to interact with Fen1 at its C-terminus (Hasan et al., 2001). The amounts of '9-1-1 complex' were the same as indicated before, for p300 fragment #4 25 µl, 50 µl and 100 µl of *E. coli* extract were used. As predicted a competition between the '9-1-1 complex' and p300 to Fen1 took place. When the amount of p300 was increased the signal of the '9-1-1 complex' on the WB decreased and vice versa (*figure 11b*). The conclusion drawn from this experiment was that the C-terminus of Fen1 is likely involved in binding to the '9-1-1 complex'.

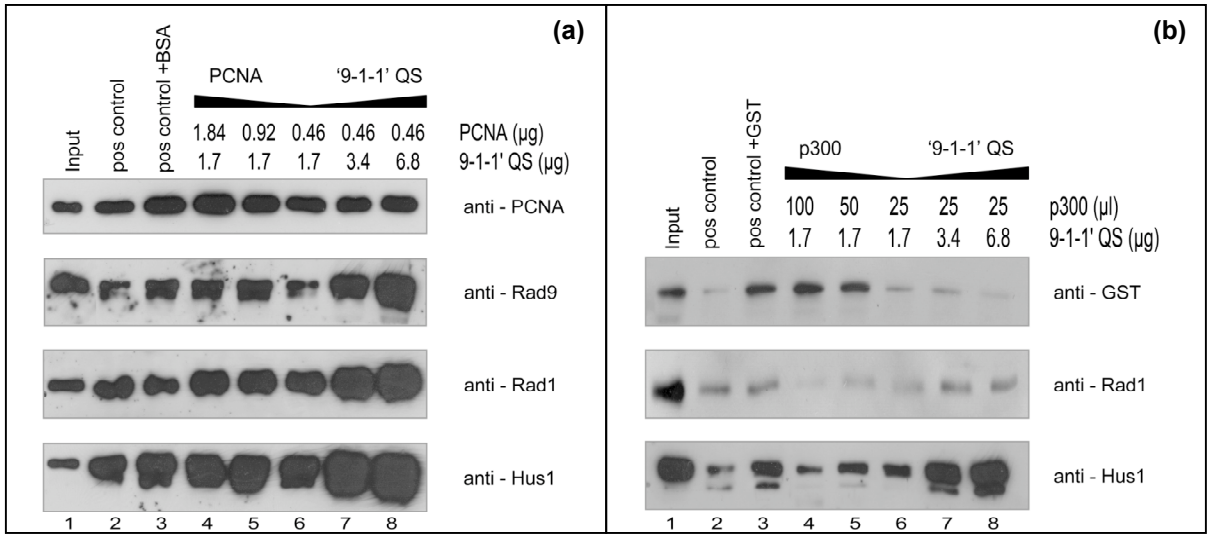

Figure 11. **p300 and the '9-1-1 complex' compete for Fen1.** For this PD competition assays were performed. **(a)** For the input (lane 1) PCNA or '9-1-1' QS was loaded. The positive controls (lane 2) show the interaction Fen1/PCNA and Fen1/'9-1-1'. In lane 3 BSA was added as a control competitor. In lanes 4 – 8 PCNA and '9-1-1' QS were added as competitors, the results were analyzed by WB developed against PCNA, Rad9, Rad1 and Hus1. **(b)** For the input (lane 1) GST p300 fragment #4 respectively '9-1-1' QS was loaded. The positive controls (lane 2) show the interaction Fen1/p300 and Fen1/'9-1-1'. In lane 3 GST was added as a control competitor. In lanes 4 – 8 p300 and '9-1-1' QS were added as competitors, the results were analyzed by WB developed against PCNA, Rad1 and Hus1. The antibody αRad9 could not be used, because it was raised against GST-Rad9 and does also crossreact with GST. A competition between '9-1-1' QS and p300 was evident.

5.5 Acetylation of Fen1 reduces the interaction with the '9-1-1 complex'

From data published from our laboratory it is known that Fen1 acetylation was enhanced upon UV treatment of human cells and was so classified as regulatory modification of Fen1. *In vitro* Fen1 was acetylated by the transcriptional coactivator p300, which forms a complex with Fen1 (Hasan et al., 2001). In mammalian cells UV damage of the DNA is basically repaired through NER, a repair mechanism into which Fen1 is not known to be involved. One would expect that acetylation of Fen1 should prevent a binding to the '9-1-1 complex', which recruits repair proteins to the UV damage site. To test this hypothesis Fen1 was acetylated *in vitro* by p300 and then tested in a PD assay with the '9-1-1 complex'. For this different histone acetyltransferases (HAT) were tested to acetylate Fen1 *in vitro* (data not shown). It turned out that purified GST p300 HAT (Bürki C. and Hottiger M. O., unpublished data) was the most efficient enzyme when the whole reaction mix was used in a subsequent PD experiment. To compensate the decreased binding of Fen1 due to the treatment during the HAT assay, 50% more Fen1 as in previous PD experiments was incubated with the Ni^{2+}-beads. To get rid of GST p300 HAT the beads were then washed with a buffer containing 500 mM NaCl, the high salt concentration did not harm the Fen1 binding to the beads (data not shown). The following steps were the same as described for PD assays before. The experiment was performed with '9-1-1 complex' QS fraction in as well as with extracts from singly infected (Rad9, Rad1, Hus1) Sf9 cells.

For the '9-1-1 complex' the signal in the WB was for all three monomers weaker when the complex interacted with acetylated Fen1 than with mock acetylated Fen1. The same results were obtained for the Rad1 and the Hus1 subunits. Only the Rad9 subunit gave the same signal for acetylated and mock acetylated Fen1 (*figure12)*. From these data it was concluded that the acetylation of Fen1 leads to a decrease of the interaction with the '9-1-1 complex'.

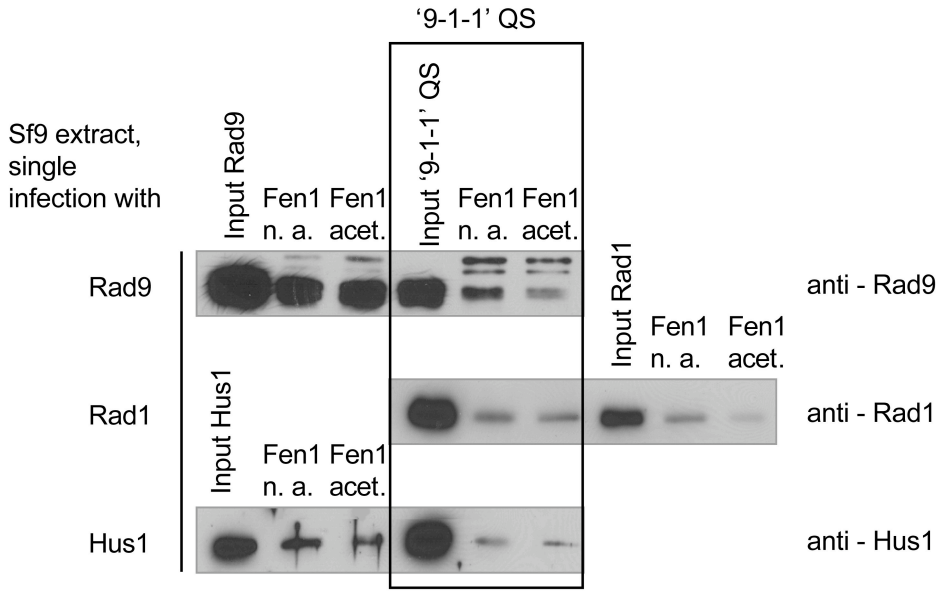

Figure 12. **Acetylation of Fen1 reduces the interaction with the '9-1-1 complex'.** A PD assay was performed. The three lanes in the middle show the input and the interaction of '9-1-1' QS with acetylated and mock acetylated Fen1wt. The signal for the binding to acetylated Fen1 is on all three panels (αRad9, αRad1 and αHus1) weaker compared to the mock acetylated. The same could also be observed for the interaction with Rad1 (middle panel at the right) and Hus1 (bottom panel at the left) from single infected cell extract. Only for Rad9 (upper panel at the left) no difference between acetylated and mock acetylated Fen1 was seen.

6 Discussion

The data presented in this thesis give evidence that the three monomers Rad9, Rad1 and Hus1 form a heterotrimeric complex in Sf9 cell extract as well as in the purified fractions. The '9-1-1 complex' is known to have structural similarity to PCNA, a functional interaction partner of Fen1. The endo- and exonuclease activity of Fen1 are highly stimulated by PCNA. Unfortunately it was not possible to measure the effect of the '9-1-1 complex' on Fen1 because a putatively co-purified nuclease interfered with the Fen1 activity. Improvement of the '9-1-1 complex' purification and further studies are required to solve this problem. What could be shown, however, was that Fen1 binds to each of the three subunits individually and also interacts directly with the '9-1-1 complex'. Three different possibilities could explain the fact that all three monomers of the '9-1-1 complex' bind to Fen1. First three Fen1 molecules can bind when Rad9, Rad1 and Hus1 appear as a complex, second the Fen1 binding site on the '9-1-1 complex' is spread over the three subunits or third, physiologically only one subunit of the complex interacts with Fen1 because the other binding sites are occupied after complex confirmation.

A competition experiment with the '9-1-1 complex' and PCNA demonstrated that the two ring-structured trimers can bind simultaneously and that they are not competing for the same interaction site on Fen1. The same experiment where PCNA was substituted by p300, a protein known to bind at the Fen1 C-terminus, showed that Fen1 C-terminus is crucial for the Fen1/9-1-1 interaction. This data was further supported by a result from a PD experiment with the Fen1 ΔC mutant, with which the '9-1-1 complex' interacted only very weakly. And again this notion is fortified by the facts that the Fen1 homologue in Archaea does not contain this C-terminus and that this early organisms do not express any checkpoint proteins. It could therefore be possible that the C-terminus of Fen1 was developed later in evolution inter alia for the interaction with checkpoint proteins.

The data of this work further support the assumption that the PCNA-like checkpoint protein '9-1-1 complex' is a novel interaction partner of Fen1. This signifies that Fen1 could be involved in the checkpoint pathway or that the '9-1-1 complex' as a checkpoint protein provides a platform for repair enzyme recruitment to DNA lesions. The observation that the '9-1-1 complex' physically interacts with pol β *in vitro* and that it has a stimulatory effect on the DNA polymerase activity (Toueille et al., submitted, 2004) strengthens this hypothesis. Pol β is a key player in BER and is known to interact with Fen1 (El-Andaloussi N. and Hottiger M.O., unpublished data). To establish the complete *in vitro* reconstitution of BER could help to elucidate the exact role of the '9-1-1 complex', Fen1, pol β and other auxiliary proteins (RF-C, PCNA, DNA ligase I).

UV damages in mammal cells are usually repaired through NER. Fen1 is not known to be involved in NER but previous studies documented that Fen1 acetylation is increased upon UV damage. Assuming that the '9-1-1 complex' is responsible for the recruitment of repair proteins, it would be logical that in case of UV damage Fen1 should not be recruited because its not involved in UV damage repair mechanism and its activity and binding capacity are reduced upon acetylation. To test if acetylation has an effect on the '9-1-1 complex' binding a pull down experiment with acetylated Fen1 was performed and the results confirmed the hypothesis. In further experiments it would be interesting to test the binding efficiency of other posttranslational modifications of Fen1 and the'9-1-1 complex' (e.g. phosphorylation (Henneke et al., 2003b)).

7 References

al-Khodairy, F. and Carr, A.M. (1992) DNA repair mutants defining G2 checkpoint pathways in *Schizosaccharomyces pombe*. *Embo J*, **11**, 1343-1350.

al-Khodairy, F., Fotou, E., Sheldrick, K.S., Griffiths, D.J., Lehmann, A.R. and Carr, A.M. (1994) Identification and characterization of new elements involved in checkpoint and feedback controls in fission yeast. *Mol Biol Cell*, **5**, 147-160.

Bermudez, V.P., Lindsey-Boltz, L.A., Cesare, A.J., Maniwa, Y., Griffith, J.D., Hurwitz, J. and Sancar, A. (2003) Loading of the human 9-1-1 checkpoint complex onto DNA by the checkpoint clamp loader hRad17-replication factor C complex in vitro. *Proc Natl Acad Sci U S A*, **100**, 1633-1638.

Bessho, T. and Sancar, A. (2000) Human DNA damage checkpoint protein hRAD9 is a 3' to 5' exonuclease. *J Biol Chem*, **275**, 7451-7454.

Burtelow, M.A., Kaufmann, S.H. and Karnitz, L.M. (2000) Retention of the human Rad9 checkpoint complex in extraction-resistant nuclear complexes after DNA damage. *J Biol Chem*, **275**, 26343-26348.

Cai, R.L., Yan-Neale, Y., Cueto, M.A., Xu, H. and Cohen, D. (2000) HDAC1, a histone deacetylase, forms a complex with Hus1 and Rad9, two G2/M checkpoint Rad proteins. *J Biol Chem*, **275**, 27909-27916.

Caspari, T., Dahlen, M., Kanter-Smoler, G., Lindsay, H.D., Hofmann, K., Papadimitriou, K., Sunnerhagen, P. and Carr, A.M. (2000) Characterization of *Schizosaccharomyces pombe* Hus1: a PCNA-related protein that associates with Rad1 and Rad9. *Mol Cell Biol*, **20**, 1254-1262.

Ceska, T.A., Sayers, J.R., Stier, G. and Suck, D. (1996) A helical arch allowing single-stranded DNA to thread through T5 5'- exonuclease. *Nature*, **382**, 90-93.

Chapados, B.R., Hosfield, D.J., Han, S., Qiu, J., Yelent, B., Shen, B. and Tainer, J.A. (2004) Structural basis for FEN-1 substrate specificity and PCNA-mediated activation in DNA replication and repair. *Cell*, **116**, 39-50.

Christmann, M., Tomicic, M.T., Roos, W.P. and Kaina, B. (2003) Mechanisms of human DNA repair: an update. *Toxicology*, **193**, 3-34.

Ellison, V. and Stillman, B. (2003) Biochemical Characterization of DNA Damage Checkpoint Complexes: Clamp Loader and Clamp Complexes with Specificity for 5' Recessed DNA. *PLoS Biol*, **1**, E33.

Enoch, T., Carr, A.M. and Nurse, P. (1992) Fission yeast genes involved in coupling mitosis to completion of DNA replication. *Genes Dev*, **6**, 2035-2046.

Fodde, R. and Smits, R. (2002) Cancer biology. A matter of dosage. *Science*, **298**, 761-763.

Foiani, M., Pellicioli, A., Lopes, M., Lucca, C., Ferrari, M., Liberi, G., Muzi Falconi, M. and Plevani, P. (2000) DNA damage checkpoints and DNA replication controls in Saccharomyces cerevisiae. *Mutat Res*, **451**, 187-196.

Friedrich-Heineken, E., Henneke, G., Ferrari, E. and Hubscher, U. (2003) The Acetylatable Lysines of Human Fen1 are Important for Endo- and Exonuclease Activities. *J Mol Biol*, **328**, 73-84.

Friedrich-Heineken, E. and Hubscher, U. (2004) The Fen1 extrahelical 3' flap pocket is conserved from archaea to human and regulates DNA substrate specificity. *Nucleic Acids Res*, **in press**.

Giannattasio, M., Lazzaro, F., Longhese, M.P., Plevani, P. and Muzi-Falconi, M. (2004) Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint. *Embo J*, **23**, 429-438.

Green, C.M., Erdjument-Bromage, H., Tempest, P. and Lowndes, N.F. (2000) A novel Rad24 checkpoint protein complex closely related to replication factor C. *Curr Biol,* **10**, 39-42.

Hang, H. and Lieberman, H.B. (2000) Physical interactions among human checkpoint control proteins HUS1p, RAD1p, and RAD9p, and implications for the regulation of cell cycle progression. *Genomics*, **65**, 24-33.

Harrington, J.J. and Lieber, M.R. (1994a) The characterization of a mammalian DNA structure-specific endonuclease. *Embo J*, **13**, 1235-1246.

Harrington, J.J. and Lieber, M.R. (1994b) Functional domains within FEN-1 and RAD2 define a family of structure- specific endonucleases: implications for nucleotide excision repair. *Genes Dev*, **8**, 1344-1355.

Hasan, S., Stucki, M., Hassa, P.O., Imhof, R., Gehrig, P., Hunziker, P., Hübscher, U. and Hottiger, M.O. (2001) Regulation of human flap endonuclease-1 activity by acetylation through the transcriptional coactivator p300. *Mol Cell*, **7**, 1221-1231.

Henneke, G., Friedrich-Heineken, E. and Hubscher, U. (2003a) Flap endonuclease 1: a novel tumour suppresser protein. *Trends Biochem Sci*, **28**, 384-390.

Henneke, G., Koundrioukoff, S. and Hubscher, U. (2003b) Phosphorylation of human Fen1 by cyclin-dependent kinase modulates its role in replication fork regulation. *Oncogene*, **22**, 4301-4313.

Hoeijmakers, J.H. (2001) Genome maintenance mechanisms for preventing cancer. *Nature*, **411**, 366-374.

Hosfield, D.J., Mol, C.D., Shen, B. and Tainer, J.A. (1998) Structure of the DNA repair and replication endonuclease and exonuclease FEN-1: coupling DNA and PCNA binding to FEN-1 activity. *Cell*, **95**, 135-146.

Hubscher, U., Maga, G. and Spadari, S. (2002) Eukaryotic DNA polymerases. *Annu Rev Biochem*, **71**, 133-163.

Hubscher, U. and Seo, Y.S. (2001) Replication of the lagging strand: a concert of at least 23 polypeptides. *Mol Cells*, **12**, 149-157.

Huggins, C.F., Chafin, D.R., Aoyagi, S., Henricksen, L.A., Bambara, R.A. and Hayes, J.J. (2002) Flap endonuclease 1 efficiently cleaves base excision repair and DNA replication intermediates assembled into nucleosomes. *Mol Cell*, **10**, 1201-1211.

Hwang, K.Y., Baek, K., Kim, H.Y. and Cho, Y. (1998) The crystal structure of flap endonuclease-1 from *Methanococcus jannaschii*. *Nat Struct Biol*, **5**, 707-713.

Jin, Y.H., Ayyagari, R., Resnick, M.A., Gordenin, D.A. and Burgers, P.M. (2003) Okazaki fragment maturation in yeast. II. Cooperation between the

polymerase and 3'-5'-exonuclease activities of Pol delta in the creation of a ligatable nick. *J Biol Chem*, **278**, 1626-1633.

Jónsson, Z.O., Hindges, R. and Hubscher, U. (1998) Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. *Embo J*, **17**, 2412-2425.

Kondo, T., Wakayama, T., Naiki, T., Matsumoto, K. and Sugimoto, K. (2001) Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. *Science*, **294**, 867-870.

Kucherlapati, M., Yang, K., Kuraguchi, M., Zhao, J., Lia, M., Heyer, J., Kane, M.F., Fan, K., Russell, R., Brown, A.M., Kneitz, B., Edelmann, W., Kolodner, R.D., Lipkin, M. and Kucherlapati, R. (2002) Haploinsufficiency of Flap endonuclease (Fen1) leads to rapid tumor progression. *Proc Natl Acad Sci USA*, **99**, 9924-9929.

Maga, G., Villani, G., Tillement, V., Stucki, M., Locatelli, G.A., Frouin, I., Spadari, S. and Hübscher, U. (2001) Okazaki fragment processing: modulation of the strand displacement activity of DNA polymerase delta by the concerted action of replication protein A, proliferating cell nuclear antigen, and flap endonuclease-1. *Proc Natl Acad Sci USA*, **98**, 14298-14303.

Majka, J. and Burgers, P.M. (2003) Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. *Proc Natl Acad Sci USA*, **100**, 2249-2254.

Melo, J. and Toczyski, D. (2002) A unified view of the DNA-damage checkpoint. *Curr Opin Cell Biol*, **14**, 237-245.

Melo, J.A., Cohen, J. and Toczyski, D.P. (2001) Two checkpoint complexes are independently recruited to sites of DNA damage in vivo. *Genes Dev*, **15**, 2809-2821.

Mossi, R. and Hubscher U. (1998) Clamping down on clamps and clamp loaders-the eukaryotic replication factor C. *Eur J Biochem*, **254**, 209-216

Murante, R.S., Rust, L. and Bambara, R.A. (1995) Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage. *J Biol Chem*, **270**, 30377-30383.

Pascucci, B., Stucki, M., Jónsson, Z.O., Dogliotti, E. and Hübscher, U. (1999) Long patch base excision repair with purified human proteins. DNA ligase I as patch size mediator for DNA polymerases delta and epsilon. *J Biol Chem*, **274**, 33696-33702.

Prasad, R., Dianov, G.L., Bohr, V.A. and Wilson, S.H. (2000) FEN1 stimulation of DNA polymerase beta mediates an excision step in mammalian long patch base excision repair. *J Biol Chem*, **275**, 4460-4466.

Reagan, M.S., Pittenger, C., Siede, W. and Friedberg, E.C. (1995) Characterization of a mutant strain of *Saccharomyces cerevisiae* with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision repair gene. *J Bacteriol*, **177**, 364-371.

Rowley, R., Subramani, S. and Young, P.G. (1992) Checkpoint controls in *Schizosaccharomyces pombe*: rad1. *Embo J*, **11**, 1335-1342.

Schurtenberger, P., Egelhaaf, S.U., Hindges, R., Maga, G., Jónsson, Z.O., May, R.P., Glatter, O. and Hübscher, U. (1998) The solution structure of functionally active human proliferating cell nuclear antigen determined by small-angle neutron scattering. *J Mol Biol*, **275**, 123-132.

Shen, B., Qiu, J., Hosfield, D. and Tainer, J.A. (1998) Flap endonuclease homologs in archaebacteria exist as independent proteins. *Trends Biochem Sci*, **23**, 171-173.

Shiomi, Y., Shinozaki, A., Nakada, D., Sugimoto, K., Usukura, J., Obuse, C. and Tsurimoto, T. (2002) Clamp and clamp loader structures of the human checkpoint protein complexes, Rad9-1-1 and Rad17-RFC. *Genes Cells*, **7**, 861-868.

Sinha, N.K., Morris, C.F. and Alberts, B.M. (1980) Efficient in vitro replication of double-stranded DNA templates by a purified T4 bacteriophage replication system. *J Biol Chem*, **255**, 4290-4293.

St Onge, R.P., Udell, C.M., Casselman, R. and Davey, S. (1999) The human G2 checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1. *Mol Biol Cell*, **10**, 1985-1995.

Storici, F., Henneke, G., Ferrari, E., Gordenin, D.A., Hübscher, U. and Resnick, M.A. (2002) The flexible loop of human FEN1 endonuclease is required for flap cleavage during DNA replication and repair. *Embo J*, **21**, 5930-5942.

Stucki, M., Jónsson, Z.O. and Hübscher, U. (2001) In eukaryotic flap endonuclease 1, the C terminus is essential for substrate binding. *J Biol Chem*, **276**, 7843-7849.

Tom, S., Henricksen, L.A. and Bambara, R.A. (2000) Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1. *J Biol Chem*, **275**, 10498-10505.

Tye, B.K. (2000) Insights into DNA replication from the third domain of life. *Proc Natl Acad Sci U S A*, **97**, 2399-2401.

Venclovas, C. and Thelen, M.P. (2000) Structure-based predictions of Rad1, Rad9, Hus1 and Rad17 participation in sliding clamp and clamp-loading complexes. *Nucleic Acids Res*, **28**, 2481-2493.

Volkmer, E. and Karnitz, L.M. (1999) Human homologs of Schizosaccharomyces pombe rad1, hus1, and rad9 form a DNA damage-responsive protein complex. *J Biol Chem*, **274**, 567-570.

Warbrick, E., Lane, D.P., Glover, D.M. and Cox, L.S. (1997) Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair. *Oncogene*, **14**, 2313-2321.

Wu, L., Davies, S.L., North, P.S., Goulaouic, H., Riou, J.F., Turley, H., Gatter, K.C. and Hickson, I.D. (2000) The Bloom's syndrome gene product interacts with topoisomerase III. *J Biol Chem*, **275**, 9636-9644.

Wu, X., Wilson, T.E. and Lieber, M.R. (1999) A role for FEN-1 in nonhomologous DNA end joining: the order of strand annealing and nucleolytic processing events. *Proc Natl Acad Sci USA*, **96**, 1303-1308.

8 Abbreviations

'9-1-1 complex'	Heterotrimeric protein complex of Rad9, Rad1 and Hus1
aa	amino acid
AcCoA	Acetyl coenzyme A
ATP	Adenosine-5'-triphosphate
Bis-Tris	Bis(2-hydroxyethyl)amino-tris-(hydroxymethyl)-methane
BSA	Bovine serum albumin
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DDT	Dithiothreitol
ECL Detection System	A non-isotopic method for detecting protein immobilised on membranes
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoresis mobility shift assay
Fen1	Flap endonuclease 1
FPLC	Fast protein, peptide and polynucleotide liquid chromatography
GroEL	Fragment of a bacterial chaperon protein
GST	Gluthadione-S-transferase
HAT	Histone acetyl transferase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid
His	Histidine
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilodalton (=1000 Dalton)
LB medium	Luria Bertani medium
LMW	Low molecular weight marker (protein marker)

NP-40	Nonidet P-40
nt	nucleotide
OD_{600nm}	Optical density measured at a wavelength of 600 nm
[γ^{32}P] ATP	ATP with radioactively labelled phosphorus in the γ position
p300	Transcriptional coactivator p300
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PD	Pull down assay
PMSF	Phenylmethylsulphonyl fluoride
Pol	DNA polymerase
Rad	Radiation resistance (proteins involved in DNA checkpoint or repair)
RF-C	Replication factor C
RNA	Ribonucleic acid
rpm	Rotor speed in revolutions per min
S. cerevisiae	*Saccharomyces cerevisiae* (budding yeast)
S. pombe	*Saccharomyces pombe* (fission yeast)
SDS	Sodiumdodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sf9 cells	*Spodoptera frugiperda* (insect) cells in culture
TBE	Tris-borate EDTA (45 mM Tris-borate (pH 8), 1 mM EDTA)
TBS	Tris-buffered saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl)
TBST	TBS with additional 0.05% Tween 20
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultra violet radiation
v/v	Volume per volume
WB	Western Blot analysis
wt	Wild type
w/v	Weight per volume

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